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CERTIFICATE

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I hereby certify that the annexed is a true copy of the Provisional Specification as filed on 15 July 1998 with an application for Letters Patent number 331002 made by Christeller, John; Sutherland, Paul; Murray, Colleen; Markwick, Ngaire and Phung, Margaret

Dated 18 August 1999.

Neville Harris
Commissioner of Patents

NEW ZEALAND

PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

IMPROVEMENTS IN OR RELATING TO CHIMERIC POLYPEPTIDES OF BIOTIN BINDING PROTEINS

WE, JOHN CHRISTELLER, a New Zealand citizen of 492 College Street, Palmerston North, New Zealand; PAUL SUTHERLAND, a New Zealand citizen of 22 Royal Terrace, Sandringham, Auckland, New Zealand; COLLEEN MURRAY, a New Zealand citizen of 6 Williams Terrace, Palmerston North, New Zealand; NGAIRE MARKWICK, a New Zealand citizen of 21 Lingham Crescent, Torbay, Auckland, New Zealand; and MARGARET PHUNG, a New Zealand citizen of 29 Juliana Place, Palmerston North, New Zealand, do hereby declare this invention to be described in the following statement:

IMPROVEMENTS IN OR RELATING TO CHIMERIC POLYPEPTIDES O BIOTIN BINDING PROTEINS

FIELD OF THE INVENTION

This invention relates to chimeric polypeptides comprising vacuole targeting sequenc and biotin binding sequences. The polypeptides are useful in methods for conferring pe resistance on plants and in the production of compositions useful as pesticides. Tl methods and compositions form further aspects of the invention.

BACKGROUND OF THE INVENTION

Pests such as insects, nematodes and mites are a significant economic cost to plant-base industries. Losses arise through production lost to pest consumption, spoilage ar introduction of disease carried by pests.

Traditionally, control of pests has been pursued through the application of pesticid chemicals. Continued use of chemicals is subject to a number of disadvantages. Pests ca develop tolerance to chemicals over time producing pesticide resistant population Chemical residues may also pose environmental hazards as well as health concerns.

Biological control presents an alternative means of pest control which is potentially mo effective and specific than current methods, as well as reducing dependence on chemic pesticides. The need for biological controls has lead to the use of recombinant DN techniques to insert genes which express pesticidal toxins into plant cells.

This technology in turn may also give rise to resistant pest populations. There is therefo an ongoing need to find proteins with pesticidal properties, particularly those that a encodes by single genes. These genes can be used to transform plants to produce pe resistant cultivars.

Genes studied to date include a range of *cry* genes from the bacterium *Bacillu thuringiensis* (Bt) encoding β-endotoxins and various higher plant genes encodin antimetabolites such as protease and α-amylase inhibitors and lectins (Boulter, 1993 Many transgenic cultivars with improved insect resistance are now being commercialise for example, transgenic cotton, corn, and potatoes (James and Krattinger, 1996). Mo recently, the use of avidin and streptavidin as larvicides against insect pests has bee

explored (WO 94/00992 and Morgan et al.; 1993). Generation of resistant plants has bee sought by inserting into the cells of a plant a gene whose expression causes production c one or more of those glycoproteins in larvicidal amounts. To date, limited success ha been achieved in producing insect resistant plants using this technology.

The commercial production of avidin from reproductive tissue of plants using such constructs has also been contemplated (WO 97/17455). The production methods are subject to a number of drawbacks. Male fertility in plants can be lost and expression in vegetative tissue may be low. This may be due in part to expression being outside the cell.

It is an object of the present invention to provide chimeric polypeptides which go some way to overcoming the above drawbacks or at least to provide the public with a useful choice.

SUMMARY OF THE INVENTION

Accordingly, in one aspect, the present invention may be broadly said to consist in a chimeric polypeptide that comprises (a) a vacuole targeting sequence encoding a polypeptide; and (b) a sequence encoding a biotin-binding protein linked in operable combination to said targeting polypeptide.

Preferably, the vacuole targeting polypeptide is a signal sequence polypeptide selected from proteinase inhibitor signal sequence (PPI-I or PPI-II) polypeptide which have the amino acid sequences set out in Figure 8b and Figure 9b respectively, or variants thereof having substantially equivalent signaling activity thereto. Numerous other signal sequences which carry out this function are described in the literature and might also be used.

Preferably, the biotin-binding protein encoded is avidin or streptavidin or a functionally equivalent variant thereof.

Conveniently, the chimeric polypeptides of the invention are obtained by expression of a DNA sequence encoding the chimeric polypeptide in a host cell or organism.

In a further aspect, the present invention provides an isolated nucleic acid molecule encoding a chimeric polypeptide of the invention.

This nucleic acid molecule can be an RNA or cDNA molecule but is preferably a DI molecule.

Also provided by the present invention are recombinant expression vectors which cont a DNA molecule of the invention, and hosts transformed with the vector of the inventi capable of expressing a polypeptide of the invention.

In a still further aspect, the invention provides a method of producing a polypeptide of i invention comprising the steps of:

(a) culturing a host cell which has been transformed or transfected with a vector defined above to express the encoded polypeptide of the invention; and optiona

(b) recovering the expressed polypeptide.

An additional aspect of the present invention provides a ligand that binds to a polypepti of the invention. Most usually, the ligand is an antibody or antibody binding fragme

In a further aspect, the present invention provides a method for producing a pest resist plant, comprising transforming the plant genome to include at least one DNA molec of the invention.

The present invention further provides a transgenic plant that contains a DNA molec of the invention.

In a still further aspect, the present invention provides a method for killing pe comprising administering to said pest an amount of a chimeric polypeptide of t invention effective to kill said pest.

Usually, the pests are insect larvae.

In yet a further aspect, the present invention provides a composition comprising chimeric polypeptide of the invention and a carrier, diluent, excipient or adjuvant.

The composition is preferably a pesticidal composition.

In a still further aspect, the present invention provides a method for producing avidin

streptavidin, the method comprising extracting avidin or streptavidin from a pl containing a DNA molecule of the invention coding for avidin or streptavidin.

While the invention is broadly as defined above, it will be appreciated by those pers skilled in the art that it is not limited thereto and that it also includes embodiments which the following description gives examples.

Figure 1 shows the nucleic acid sequence of Potato Proteinase Inhibitor I (PPI-I/pUC1 The signal sequence is in bold type and the start and stop codons are in italic. T mutagenic primer is denoted by underlined in lower case with the Bgl II site created mutagenesis in bold italic. The upstream and downstream primers used were the Forw and Reverse M13(lacZ) Primers [Perkin Elmer].

Figure 2 shows Avidin cDNA (pGEMav). The signal sequence represented in bold ty start and stop codons are in italic, primers are underlined lower case with the BamH I s created by mutagenesis in italic. The downstream primer used was the Reve M13(lacZ) Primer [Perkin Elmer].

Figure 3 shows streptavidin cDNA (Streptavidin/pUC19). Start and stop codons are bold type. EcoR I and Xba I sites are in italic.

Figure 4 shows potato proteinase inhibitor II (PPI-II/pUC19). The signal sequence represented in bold type and start and stop codons are in bold italic. Underlined ty denotes the intron within the signal sequence. The asterisk denotes the result of PCR en during isolation of the PPI-II sequence.

Figure 5 shows components of the ligation reaction to produce recombinant pAR containing the PPI-I signal sequence/Avidin cDNA gene fusion. A) PPI-I leader fragme resulting from a Sal I/Bg1 II digest of the mutated PPI-I PCR product. B) Avidin matu protein cDNA fragment, resulting from a BamH I/Hind III digest of the mutated Avic PCR product. C) pART7 vector following an Xho I/Hind III digestion. * denot compatible cohesive ends. ** denotes compatible cohesive ends.

Figure 6 shows DNA fragments A, B and C were the components of the ligation reacti to produce recombinant pUC19 containing the PPI-II signal sequence/Streptavidin cDN gene fusion. The fused gene was then released from pUC19 by a Sal I/BamH I digest a ligation of components D and E produced recombinant pART7. A) PPI-II leader fragme

resulting from a Sal I/EcoR I digest of the PPI-II PCR product. B) Streptavidin cDNA fragment, resulting from an EcoR I/Xba I digest of the recombinant plasmid pUC19/Streptavidin cDNA. D) PPI-II signal sequence/Streptavidin cDNA gene fusion fragment, resulting from a Sal/BamH I digest of recombinant pUC19 containing the fused gene. E) pART7 vector following an Xho I/BamH digestion. * denotes compatible cohesive ends.

Figure 7 shows a schematic representation of the pART7 expression cassette as it was cloned into the pART27 binary vector; A) containing the PPI-I-Avidin gene fusion and B) containing the PPI-II/Streptavidin gene fusion.

Figure 8 shows PPI-I/Avidin gene fusion sequence (A) and fusion protein sequence (B): The fusion protein has a total of 161 amino acids; the PPI-I sequence is represented by italic type with bold type denoting the PPI-I signal peptide. Two amino acids, novel to both the PPI-I and the Avidin peptide sequences and represented in lower case were introduced with the ligation of the Bgl II and BamH I compatible cohesive ends.

Figure 9 shows PPI-II/Streptavidin gene fusion sequence (A) and fusion protein sequence (B): The fusion protein has a total of 168 amino acids; the PPI-II sequence is represented by italic type with bold type denoting the PPI-II signal peptide. Three amino acids, novel to both PPI-II and the Streptavidin peptide sequences and represented in lower case were introduced at the point of fusion.

Figures 10 and 11 show the mortality of potato tuber moth larvae on whole tobacco plants expressing the avidin gene in two replicate trials.

Figure 12 (A) shows nucleotide sequence for the gene for streptavidin (Argarana C.E. et al. 1986). The signal sequence is represented in bold type, start and stop codons in bold italic. (B) shows protein sequence for streptavidin the signal sequence is represented in bold type.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel chimeric polypeptides comprising vacuole targeting sequences and biotin-binding sequences. The targeting sequences and biotin binding sequences are operably linked.

The term "operably linked" as used herein refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a signal sequence is operably linked to a coding sequence if the promoter affects its transcription or expression.

The vacuolar targeting polypeptide sequences of the invention, when transformed into plants, function to direct the protein products directed by the expression of genes to which they are operably linked from the cytoplasm to the vacuole of the plant cell. Since the vacuole of plant cells has a storage function, proteins directed there remain there, continually increasing in abundance, unless subject to degradation by vacuolar proteinases. The vacuolar proteins are also isolated from the major metabolic processes in the plant and thus will not interfere with the plant growth and development. The success of the present invention required that both these requirements be met.

Vacuolar targeting sequences include any such targeting sequences as are known in the art. These include polypeptides targeting barley lectin (Bednarek et al., 1990)[3], sweet potato sporamin (Matsuoka et al., 1990)[16], tobacco chitinase (Neuhaus et al., 1991)[23], bean phytohemagglutinin (Tague et al., 1990)[28], 2S albumin (Saalbach G et al., 1996)[24], aleurain (Holwerda et al., 1992)[14]. See also references 7, 8, 13, 25, 33, 32 and 35-46 referenced on pages 27 to 31. However, potato proteinase inhibitor targeting sequences are preferred.

A number of potato proteinase signal sequence polypeptides designated PPI-I and PPI-II are disclosed herein. The polypeptides have the amino acid sequences set out in Figures 8b and 9b respectively. Also encompassed within the invention are variants of these polypeptides and those known in the art which have substantially equivalent targeting sequence activity thereto.

The term "variant" as used herein refers to a polypeptide wherein the amino acid sequence exhibits substantially 70% or greater homology with the amino acid sequences set out in Figures 1 and 4. Preferably, the variants will have greater than 85% homology, and most preferably, 95% homology or more. Variants may be arrived at by modification of the native amino acid sequence by such modifications as insertion, substitution or deletion of one or more amino acids.

As noted above, chimeric polypeptide comprising a vacuolar targeting signal sequence operably linked to a biotin-binding protein. Biotin is an essential nutrient for many

species of pests (Dadd, R.H., (1985)[9]. Nutrition: Organisms In: Comprehensive In Physiology, Biochemistry and Pharmacology (Kerkut G.A. and Gilbert, L.L.) Prega Press, NY Vol 4, p313-390). As discussed above, biotin-binding proteins have been f to have pesticidal properties and to inhibit growth of pests. The binding of biotin ca a biotin deficiency which results in the inhibition of growth and ultimate death of p

The term "pest" as used herein refers to a broad group of organisms which have a b requirement, including protozoa, arthropods (especially insects), aschelmin platyhelminthes, nematodes and molluscs.

Biotin-binding proteins known in the art include egg yolk (Subramanian & Adiga, 19 serum (Seshagiri & Adiga, 1987)[26], and bacterial proteins, avidin, isolated from egg w and streptavidin.

Preferred biotin-binding polypeptides, for use in the present invention, are avidin streptavidin or functionally equivalent variants thereof. It will be appreciated that c proteins that function to bind biotin are equally able to be used in the present inven

Avidin is a water-soluble tetrameric glycoprotein isolated originally from raw egg w (J. Biol.Chem 136: 801 (1940)). The protein is well known with the complete amino sequence having been published in, for example, J.Biol.Chem. 246: 698 (1971). The amino acid sequence for avidin is shown in Figure 8b (amino acids 34 to 161).

Streptavidin is a non-glycosylated bacterial binding protein derived from the cu supernatant of *Streptomyces avidinii* (Bayer, E.A., et al., 1990)[2]. The full amino sequence for streptavidin is given in Figure 12.

'Core' SAV is equivalent to amino acid residues 37-164 of *Streptomyces avidinii* (S Figure 12, (Argarana et al., (1986))[1]. A preferred sequence referred to as "Synthetic 'C Streptavidin" is a modified 'core' SAV having the sequence shown in Figure 9b (ar acids 41 to 168). SYNSAV is equivalent to 'Core' SAV modified such that codon each amino correspond to those in highly expressed E.coli genes. SYNSAV is modified to contain unique restriction sites evenly throughout sequence. The resu sequence has G + C content of 54% relative to 69% for same region of native S (Thompson, L.D., et al. (1993))[29].

The reader will appreciate that modifications, including chemical and biochen

modifications, of the polypeptides of the invention are possible. Such modifications include, for example, acetylation, carboxylation, phosphorylation, glycosylation, ubiquitination, labeling, and the like. The production of peptide fragments is also well within the capabilities of an art skilled worker.

The polypeptides of the invention can be prepared in a variety of ways. For example, as indicated above for the signal sequences and biotin-binding proteins can be produced by isolation from natural sources and then coupled using techniques known in the art. For example, through recombinant nucleic acid methods.

Synthesis using known techniques (such as stepwise solid phase synthesis described by Merryfield (1963), *J. Amer.Chem.Soc.* Vol 85:2149-2156), or as preferred through employing recombinant DNA techniques.

The variants of both the polypeptide and peptides can similarly be made by any of those techniques known in the art. For example, variants can be prepared by site-specific mutagenesis of the DNA encoding the native amino acid sequence as described by Adelman et al. *DNA* 2:183 (1983).

Where it is preferred, recombinant techniques used to produce the polypeptide or peptide of the invention, the first step is to obtain DNA encoding the desired product. Such DNA comprises a still further aspect of this invention.

The DNA of the invention may encode a native or modified polypeptide or peptide of the invention or an active fragment thereof. In its presently preferred forms, the DNA comprises the nucleotide sequence of Figure 8A, or the nucleotide sequence of Figure 9A. Preferred sequences exhibit 60% or greater homology with these sequences, preferably 80% homology and most preferably 95% homology or more. That is, most preferred sequences will hybridise to the sequences of the invention under stringent hybridisation conditions.

The DNA can be isolated from any appropriate natural source or can be produced as intron free cDNA using conventional techniques. DNA can also be produced in the form of synthetic oligonucleotides where the size of the active fragment to be produced permits. By way of example, the Triester method of Matteucci et al. *J. Am.Chem.Soc.* Vol 103:3185-3191 (1981) may be employed.

Where desirable, the DNA of the invention can also code for the chimeric polypeptid the invention. Fusion proteins further comprising the polypeptide or peptide of invention and a carrier protein are possible. This carrier protein will generally cleavable from the polypeptide, peptide or fragment under controlled conditi Examples of commonly employed carrier proteins are β-galactosidase and glutathion transferase.

As indicated above, also possible are variants of the polypeptide or peptide which di from the native amino acid sequence by insertion, substitution or deletion of one or m amino acids. Where such a variant is desired, the nucleotide sequence of the native D is altered appropriately. This alteration can be made through elective synthesis of DNA or by modification of the native DNA by, for example, site-specific or cass mutagenesis. Preferably, where portions of cDNA or genomic DNA require seque modifications, site-specific primer directed mutagenesis is employed using techniq standard in the art.

In a further aspect, the present invention consists in replicable transfer vectors suitable use in preparing a polypeptide or peptide of the invention. These vectors may constructed according to techniques well known in the art, or may be selected fr cloning vectors available in the art.

The cloning vector may be selected according to the host or host cell to be used. Use vectors will generally have the following characteristics:

(a) the ability to self-replicate;
(b) the possession of a single target for any particular restriction endonuclease; an
(c) desirably, carry genes for a readily selectable marker such as antibiotic resistan or herbicide tolerance.

Two major types of vector possessing these characteristics are plasmids and bacter viruses (bacteriophages or phages). Presently preferred vectors include the plasmi pMOS-Blue, pGem-T, pUC18, pUC19, pART27, pMON, pJIT, pBIN, pRD 400, pAR

The DNA molecules of the invention may be expressed by placing them in operat linkage with suitable control sequences in a replicable expression vector. Cont sequences may include origins of replication, a promoter, enhancer and transcriptio terminator sequences amongst others. The selection of the control sequence to

included in the expression vector is dependent on the type of host or host cell intended be used for expressing the DNA.

Generally, procaryotic, yeast, insect or mammalian cells are useful hosts. Also include within the term hosts are plasmid vectors. Suitable procaryotic hosts include E. col Bacillus species and various species of Pseudomonas. Commonly used promoters suc as β-lactamase (penicillinase) and lactose (lac) promoter systems are all well known i the art. Any available promoter system compatible with the host of choice can be used Vectors used in yeast are also available and well known. A suitable example is the micron origin of replication plasmid.

Similarly, vectors for use in mammalian cells are also well known. Such vectors include well known derivatives of SV-40, adenovirus, retrovirus-derived DNA sequences, Herpes simplex viruses, and vectors derived from a combination of plasmid and phage DNA.

Further eucaryotic expression vectors are known in the art (e.g. P.J. Southern and P.Berg, *J. Mol. Appl. Genet. 1* 327-341 (1982); S. Subramani et al., *Mol.Cell.Biol. 1*, 854-864 (1981); R J. Kaufmann and P.A. Sharp, "Amplification and Expression of Sequences Cotransfected with a Modular Dihydrofolate Reducase Complementary DNA Gene, *J. Mol. Biol. 159*, 601-621 (1982); R J. Kaufmann and P.A. Sharp, *Mol.Cell.Biol. 159*, 601-664(1982); S.I. Scahill et al., "Expressions And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," *Proc. Natl. Acad. Sci. USA. 80*, 4654-4659 (1983); G. Urlaub and L.A. Chasin, *Proc. Natl. Acad. Sci. USA. 77*, 4216-4220, (1980).

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the glycolytic promoters of yeast acid phosphatase, e.g. Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g. the early and late promoters of SV40, and other sequences known to control the expression of genes of prokaryotic and eucaryotic cells and their viruses or combinations thereof.

Preferred promoters for use herein include lacZ, CaMV-35S, LHC a/b, T7, nos, rubisco

small subunit (SSU), gpd and nod gene promoters.

In the construction of a vector it is also an advantage to be able to distinguish the ve incorporating the foreign DNA from unmodified vectors by a convenient and rapid as Such assays include measurable colour changes, antibiotic resistance, herbicide toler and the like. In one preferred vector, the β-galactosidase gene is used, which ge detectable by clones exhibiting a blue phenotype on X-gal plates. This facili selection.

Once selected, the vectors may be isolated from the culture using routine procedures s as freeze-thaw extraction followed by purification.

For expression, vectors containing the DNA of the invention to be expressed and cor signals are inserted or transformed into a host or host cell. Intermediate host cells ca used to increase the copy number of the cloning vector prior to introduction into p cells. Some useful expression host cells include well-known prokaryotic and eucary cells. Some suitable prokaryotic hosts include, for example, E.coli, such as E. coli, S 936, E. coli HB 101, E. coli W3110, E.coli X1776, E. coli, X2282, E. coli, DHT, an coli, MR01, Pseudomonas, Bacillus, such as Bacillus subtilis, and Streptomyces. Suit eucaryotic cells include yeast and other fungi, insect, animal calls, such as COS cells CHO cells, human cells and plant cells in tissue culture.

Expression systems employing insect cells utilising the control systems provided baculovirus vectors have been described (Miller, D W et al., in *Genetic Enginee* (1986) Setlo W, J K et al., Eds, Plenum Publishing, Vol 8, pages 277-297).

Depending on the host used, transformation is performed according to standard techniq appropriate to such cells. For prokaryotes or other cells that contain substantial cell w the calcium treatment process (Cohen, S N *Proceedings, National Academy of Scie USA* 69 2110 (1972)) may be employed. For mammalian cells without such cell walls calcium phosphate precipitation method of Graeme and Van Der Eb, *Virology* 52:: (1978) is preferred. Transformations into plants may be carried out using *Agrobacter tumefaciens* (Shaw et al., Gene 23:315 (1983) or into yeast according to the metho Van Solingen et al. *J.Bact. 130*: 946 (1977) and Hsiao et al. *Proceedings, Natio Academy of Science*, 76: 3829 (1979).

In a preferred transformation process, the vectors of the invention are incorporated i

Agrobacterium tumefaciens which can be used to infect plant cells, partici dicotyledenous plant cells, thereby transferring the vectors and conferring pest resist The cloning vectors can also be introduced into plant cells using convenient art techn such as electroporation, microparticle bombardment and microinjection. Micropa bombardment is the preferred transformation process for monocotyledenous p Suitable plant transformation techniques are usefully summarised in Torres et al., Cell, Tissue and Organ Culture 34: 279-285 (1993), Michelmore et al., Plant Cell Re 6:439-442 (1987), Horsch et al., Plant Molecular Biology Manual AS: 1-9 (1988), X et al., J. Genet. and Breed. 46: 287-290 (1992) and WO 97/17455 incorporated here reference.

Upon transformation of the selected host with an appropriate vector the polype encoded can be produced, often in the form of fusion protein, by culturing the host The polypeptide of the invention may be detected by rapid assays as indicated above. polypeptide can then be recovered and purified if desired. Recovery and purificatio be achieved using any of those procedures known in the art, for example by absor onto and elution from an anion exchange resin. This method of producing a polype of the invention constitutes a further aspect of the present invention.

The present invention also provides a method for producing avidin or streptavidin method comprising extracting avidin or streptavidin from a plant incorporating a I sequence of the invention coding for avidin or streptavidin. The expression level of a or streptavidin may be increased by further incorporating into the DNA sequence o invention a peptide export signal sequence, or intron sequence. Methods of enhan expression levels and method for production of avidin and streptavidin generally ma effected according to the techniques of WO 97/17455 incorporated herein by refere The use of the chimeric polypeptides of the present invention represents an advance this document because the avidin or streptavidin is produced in vegetative tissues (le stems, tubers, roots) as opposed to the reproductive tissues. The method can be use avidin or streptavidin from a wide range of plants which produce abundant veget material e.g. potatoes, cassava, tobacco, grasses, legumes, and trees rather than b restricted to plants which produce large reproductive structures e.g. maize.

Plants suitable for transformation with the vectors of the invention may be selected a broad range of plants including cereal crops, vegetable, fruit and other food crops, fo crops and turf plants, fibre crops, timber and pulp and paper plants, shelter-belt plants tree crops, ornamental and flower plants, culinary plants, medicinal plants and herbs

plants grown to produce beverages.

Examples of cereal crops include wheat, rice, barley, maize, oats, millet, sorghum and

Examples of vegetable, fruit and other food crops include root crops such as potato, s potato, beetroot, parsnip, turnip, swede and carrot, cucurbits such as cucuml pumpkins, squash, marrow, courgettes and watermelon, brassicas such as cauliflo cabbage, oilseed rape, brussels sprouts and broccoli, corn, tomato, lettuce, celery, oni garlic, legumes such as lentils, green beans, lima beans, haricot beans, red kidney be kudzu beans, mung beans, broadbeans, soybeans, chickpeas, peas, and peanuts, ap pear, kiwifruit, tamarillo, feijoa apricot, plum, citrus such as orange, lemon, tang grapefruit, uglifruit and mandarin, pineapple, peach, nectarine, cherry, berries, olives sugarcane.

Examples of forage crops and turf plants include legumes such as clover, alfalfa, l trefoil and lucerne and grasses and other graminaceous plants such as ryegrass, brown fescue, cocksfoot, kikuyu and, paspalum, and sorghum grass.

Fibre crops include cotton, flax, kapok and hemp. Timber, shelterbelt, conservation, and paper plants and tree crops include, for example, pine, eucalyptus, spruce, fir, ash, birch, beech, mahogany, rosewood, ebony, maple, teak, cedar, redwood, jar chestnut, walnut, macadamia nut, poplar, willow, cypress, camphor, mulberry, mar grass and rubberplant.

Ornamental shrubs, trees and flower plants include roses, petunias, orchids, carnati chrysanthemums, daisies, tulips, lilies, gypsophylla, hibiscus, rhododendrons, coni camellias, hebes, lavender, lupins, tussock, ferns and native plants. Culinary pl include herbs such as basil, rosemary, oregano, bay, and spices such as cinnamon, m tumeric, and sage.

Medicinal plants include poroporo, opium poppies, coca, marijuana, camomile, coml foxglove, belladonna.

Plants used to produce beverages include tea, coffee, hops and cocoa.

Plants transformed with the vectors of the invention direct expression of the biotin bin proteins in the vacuoles of the plant cells. The biotin binding protein is effecti

sequestered into the vacuole. When an insect pest feeds on the plant, the plant cel components mix together allowing biotin to be bound by the binding protein. This essentially deprives the insect of the vitamin it requires leading to stunted growth and death.

The present invention has application in producing plants resistant to a broad range of pests in adult or larval stage including moths, beetles, weevils, caterpillars, borers, budworms, armyworms, bollworms, rootworms, webworms, aphids, bugs, crickets, locusts, grubs, flies, fruitflies, leafminers, plant hoppers, earwigs, scale insects, thrips, and springtails. Plants of the invention may also be resistant to other invertebrate pests of plants such as slugs, snails, mites, lice and nematodes and other pests and pathogens which have a vitamin requirement for biotin.

Accordingly, in a further aspect the invention provides a method of imparting pest resistance to plants comprising transforming the plants with a vector according to the present invention.

The method may also be effected by transforming isolated plant cells or tissues and generating plants from the transformed cells or tissue using standard culture techniques. Plant cells and tissue cultures transformed with vectors of the invention form further aspects of the invention.

Transformed plants can be used in conventional breeding programmes to transfer the DNA sequences of the invention.

In another aspect, the present invention also provides a composition comprising a chimeric polypeptide of the invention and a carrier diluent, excipient or adjuvant therefor.

Preferably, the composition is a pesticidal composition comprising a pesticidally effective amount of the polypeptide and an acceptable carrier. The pesticidal composition can be applied to plants in the form of sprays, dusts, or other formulations commonly employed in making pesticides.

In another embodiment, the composition may be applied to harvested material to prevent pest damage in storage. In an extrapolated application, the compositions may similarly be used in plant derived products such as flours, meals, cereals and the like to prevent or control pest infestation.

It will be appreciated that the above description is provided by way of example only an that variations in both the materials and techniques used which are known to those person skilled in the art are contemplated.

Non-limiting examples illustrating the invention will now be provided.

EXAMPLE 1

Materials

Custom primers were synthesized by Life Technologies. Subcloning Efficiency DH5 competent Cells were purchased from Life Technologies and the Hybaid Recovery Plasmid Mini Prep Kit from Hybaid Limited. All enzymes, unless otherwise stated were purchased from Promega. Ampligase Thermostable DNA Ligase and Reaction Buffer and GELase were purchased from Epicentre Technologies and Polymerase Chain Reaction (PCR) reagents from Perkin Elmer.

The Avidin cDNA (pGEMav) carried on the plasmid pGEM3 was supplied by Professor M. S. Kulomaa ((Department of Biological and Environmental Science, University of Jyvaskyla, Finland) and the Potato Proteinase Inhibitor I (PPI-I) cDNA was isolated in this laboratory (Beuning et al. 1994, GenBank Accession # L06606) and cloned into pUC19.

The Streptavidin cDNA, carried on the plasmid pET3a was supplied by The DuPont Merck Pharmaceutical Company. The Potato Proteinase Inhibitor II (PPI-II) genomic sequence was isolated in this laboratory and cloned into pUC19 (Murray C. and Christeller J. T. 1994)[21].

Methods

Subcloning Efficiency DH5 competent Cells were used for general cloning and amplification of recombinant plasmids and the Hybaid Recovery Plasmid Mini Prep Kit was used for plasmid preps. Isolation and recovery of DNA fragments was achieved by agarose gel electrophoresis followed by treatment of excised gel bands with GELase.

DNA Sequencing and Computer Analysis:

DNA sequencing was carried out on an Applied Biosystems (ABI) DNA Sequencer using dye terminator chemistry. Sequence analysis was performed using the Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin.

EXAMPLE 2

Preparation of a binary vector designed to express a chimeric polypeptide comprising Avidin mature peptide fused to a Potato Proteinase Inhibitor I Sig Peptide

Methods

A one-step PCR-based mutagenesis method employing the combined use o thermostable DNA polymerase and thermostable DNA ligase (Moore D.S. and Mich S. F. 1995)[19], was used to prepare a construct comprising the sequence encoding mature Avidin polypeptide (Gope M.L. et al. 1987)[12] fused to a PPI-I signal sequence Bgl II site was produced downstream of the PPI-I leader sequence at positions 92 - 97 the PPI-I coding sequence and a BamH I site was created upstream of the sequer encoding the mature Avidin polypeptide, at positions 65 - 70 of the sequence encoding Avidin protein, as shown in Fig. 1 and Fig. 2 respectively. These two restriction sites ha compatible cohesive ends.

Primers:

Forward M13 (lacZ) Primer [Perkin Elmer]:

5'-GCCAGGGTTTTCCCAGTCACGA-3'

Reverse M13 (lacZ) Primer [Perkin Elmer]:

5'-GAGCGGATAACAATTTCACACAGG-3'

Avidin Upstream Primer:

5'-GCACACCCGGCTGTCCACCTG-3'

Phosphorylated Mutagenic Primers

PPI-I mutagenic primer:

5'-PGATGGACCAGAGATCTTAGAAC-3'

Avidin mutagenic primer:

5'-PGGCTCCCGGGATCCCTGCCAG-3'

Amplification/Mutagenesis reactions

To generate mutant products a total PCR reaction volume of 50 ul with an effective Ampligase Reaction Buffer [20 mM Tris-HCl (pH 8.3 at 25°C), 25 mM KCl, 10 $MgCl_2$, 0.5 mM NAD and 0.01% Triton X-100] was used with the following conditi

100 pmol each outer primer
1 nmol phosphorylated mutagenic primer
40 nmol each dNTP
0.1 umol dithiothreitol
5 U Taq DNA polymerase
5 U thermostable DNA ligase
1ng recombinant plasmid DNA template

Reactions were first incubated at 94°C for 3 min., followed by 30 amplification cy performed as follows:

94°C, 1 min.
40°C, 1 min.
65°C, 6 min.

Amplification cycles were followed by a final extension at 65°C for 7 min.

Restriction analysis of amplification products from both mutagenesis reactions revea mutant product to be present, but only at a maximum of 5% of the total product. increase the yield of mutated product, Bgl II (for PPI-I mutagenesis) and BamH I (Avidin mutagenesis) digestion products were ligated and then used as template fo second amplification reaction using outer primers only (Avidin Upstream and Reve M13 (lacZ) for AVIDIN; Forward M13 (lacZ) and Reverse M13 (lacZ) for PPI-1). PPI-I, greater than 95% of second round amplification product had the desired Bgl II and approximately 80% of the second round product for Avidin mutagenesis posses the BamH I site.

The mutated PPI-I amplification product was digested with Bgl II and Sal I and mutated Avidin product with BamH I and Hind III. The PPI-I leader sequence and coding sequence for the Avidin mature protein were isolated and recovered for clon

along with Xho I/Hind III digested non-recombinant pART7 vector (Gleave A.P.1992)[1] These three species were ligated, resulting in recombinant pART 7 [refer Fig. 5] and th sequence of the chimeric gene was checked. Subsequently, the expression cartridg containing the gene fusion was cloned into the Not I site of pART27 vector (Gleav A.P.1992)[11] and this construct [refer Fig. 7A] was mobilized to *Agrobacteriur tumefaciens* (strain LBA4404) by standard tri-parental mating techniques (Ditta G. et al 1980)[10].

Discussion

The resulting PPI-I/Avidin fusion protein has a total of 161 amino acids as shown in Fig. 8. The first 31 amino acids are PPI-I sequence and since the leader sequence comprises the first 23 amino acids, the original patterning of amino acids around with the site for cleavage between the signal sequence and the mature protein is retained. There are two single base pair changes in the gene fusion sequence relative to the predicted sequence. These changes are presumably the result of PCR error. One change is silent and the other results in an amino acid change from Serine to Proline at position 17 of the PPI-I signal sequence.

EXAMPLE 3

Preparation of a binary vector designed to express a chimeric polypeptide comprising Synthetic "Core" Streptavidin peptide fused to a Potato Proteinase Inhibitor II Signal Peptide

Methods

A fused gene was prepared comprising the sequence encoding Synthetic "Core" Streptavidin (Thompson L. D. and Weber P. C. 1993)[29] fused to a PPI-II signal sequence. The Streptavidin cDNA, carried on the plasmid pET3a was cloned into the EcoR I/Xba I sites of pUC 19 (Fig. 3). The PPI-II signal sequence (Fig. 4) which contains an intron was isolated from recombinant plasmid using PCR with a sense primer binding to pUC19 and an antisense primer incorporating an EcoR I site into a 5' overhang. The primers were as follows:

sense primer:

5' - CTG CAG GTC GAC TCT AGA GGA - 3'

antisense primer:

5' - GGT GAA TTC TTA GTA CAG ATC TTC GCA - 3'

Amplification reaction

A total PCR reaction volume of 50 ul with an effective 1 X PCR Buffer [10 mM Tris-H pH 8.3 and 50 mM KCL] was used with the following conditions:

20 pmol each primer
15 nmol each dNTP
2.0 mM $MgCl_2$
5 U Taq DNA polymerase
1ng recombinant plasmid DNA template

Reactions were first incubated at 94°C for 2 min., followed by 30 amplification cyc performed as follows:

94°C, 1 min.
50°C, 1 min.
72°C, 1 min.

Amplification cycles were followed by a final extension at 72°C for 7 min.

The PCR product representing the PPI-II signal sequence was digested with Sal I a EcoR I. The recombinant plasmid pUC 19/Streptavidin cDNA was digested with Ec I and Xba I and the Streptavidin cDNA was isolated from the vector and recovered. Nc recombinant pUC19 was digested with Sal I and Xba I and the three species were ligat to produce a construct comprising the gene fusion cloned into the Sal I and Xba I sites pUC19. The sequence of the gene fusion was checked and subsequently cloned into t Xho I and BamH I sites of the pART7 vector [refer Fig. 6]. The pART7 expressi cartridge containing the gene fusion was then cloned into the Not I site of pART27 a this construct [refer Fig. 7B] was mobilized to *Agrobacterium tumefaciens* (str LBA4404) by standard tri-parental mating techniques.

Discussion

The resulting PPI-II/Streptavidin fusion protein has a total of 168 amino acids as sho in Fig. 9. The first 36 amino acids are PPI-II sequence. Five of these amino acids foll the cleavage site, preserving the amino acid pattern around this position. The nucleoti sequence of the PPI-II signal sequence includes a 119 bp intron (Murray C. and Christel J. T. 1994)[21].

EXAMPLE 4
Immunodetection of avidin in transgenic tobacco

Methods

1. Tissue print

Pieces of transgenic tobacco leaves 1 x 1cm were frozen at -20 C for 20 min, allowed to thaw and printed on to nitrocellulose using mechanical pressure. The printed nitrocellulose membrane was washed in PBS-T (phosphate buffered saline with 0.1% Tween 20) for 20 min, blocked in 0.1% BSA-C (Aurion) for 15min and incubated in 1:1000 anti-avidin (Sigma A-5170) diluted in blocking buffer for 1h (This last step is deleted for control). The membrane was then washed in PBS-T, incubated in goat anti-rabbit IgG-gold (10nm) (Sigma), washed again in PBS-T, then in ddH_20 and drained. Finally the membrane was silver enhanced (BioCell silver enhancement kit) for 15min.

2. Embedded material

Pieces 1 x 1x 5mm of transgenic tobacco leaf were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer under vacuum for 1h. The material was post-fixed in 1% osmium tetroxide 1h, dehydrated in an ethanol series and embedded in Spurrs resin. Sections were cut 1μm thick for light microscopy (LM) and mounted on Poly-L-lysine coated slides. Sections for electron microscopy (EM) were cut 130nm thick (gold) and mounted on carbon/formvar coated nickel grids.

For light microscopy the sections had a Pap pen ring drawn around them to contain the incubation liquid. The protocol for LM and EM were the same thereafter. The sections were etched for 30min in 10% hydrogen peroxide to remove the osmium, blocked in 0.1% BSA-c for15 min, incubated in anti-avidin 1:100 in PBS-T for 1h and washed in PBS-T. They were then incubated in goat anti-rabbit IgG-gold (10nm) for 1h, washed in buffer, then water and finally silver enhanced for 7 min for the LM section and counter stained with toluidine blue.

Results

The nitrocellulose membrane silver enhanced (turned brown) over the entire area of the tissue print. There was no silver enhancement on the control print. This labelling protocol also acts as a test of the labelling procedure.

Immunolabelling of LM and EM sections showed labelling of protein-type bodies in the

vacuoles of mesophyll cells (both spongy and pallisade) and in glandular hairs. protein bodies were usually condensed into one body which was sometimes seen as a r There was a lower level of labelling in the cytoplasm.

Conclusions

The results indicate that avidin is synthesized in most common cell types in toba leaves. The bulk of the protein appears to be transported to the vacuole and deposite a protein body within this organelle.

EXAMPLE 5

Feeding trials with neonate potato tuber moth larvae on whole tobacco pla expressing avidin

Constructs:

Non-transformed control plants

- 2 plants (NT 1, NT 2)

Control plants transformed with pumpkin fruit chymotrypsin inhibitor (PFCI) but expressing the protein

- 3 plants (JB3/1, J13312, JB5/1)

Tobacco plants transformed with the avidin gene with a PPI-1 targeting sequence(exam 2 above)

- 6 lines line (PLA2/2, PLA2/7, PLA2/9, PLA2/13, PLA2/20, PLA2/24), 4 clo plants per line.

Trial Design

Trial i

The tobacco plants were removed from tissue culture and potted in fertilised potting n (Smiths ® general potting mix) before being placed in large ventilated acetate contain (220 x 300mm) in a containment glasshouse unit at 22±5 C. They were watered daily maintain high humidity and soil moisture content.

Eight days later, when plants were well established with at least 4-5 small leaves, t neonate potato tuber moth (PTM) larvae were placed on each tobacco plant, usually t per leaf. Prior to inoculation the larvae were weighed in batches of five (since sing larvae are too small to give an accurate reading). TM larvae were obtained from

laboratory culture reared on potato tubers following the same basic procedure as Brooc (1971)[6] and Meisner et al. (1974)[17].

Trial ii

One week after Trial i was completed, the tobacco plants were cut back to the second n and allowed to regenerate leaves. When the plants had developed 4-5 leaves approximately 11d) they were each inoculated again with ten neonate PTM larvae, usu 2 per leaf, weighed in batches of five prior to inoculation as above.

Trials i and ii

Inoculated plants were kept individually in acetate containers in the containm glasshouse unit at 22±5 C for nine days. Under these conditions growth of control lar is exponential from hatch to nine days, but after this growth rate slows as pupat approaches. Hence in order to compare growth rates of larvae on control and transge plants, the trial was concluded after nine days. Damaged leaves containing larvae w removed and photographed, and larvae were dissected out of their mines within the l or stem tissue. The intention was to weigh the larvae at this point in order to estim growth rates, but, except for those on control plants, larvae were mostly dead, dried a shrivelled. Consequently head capsules were measured so that the instar reached at de could be recorded.

Results

Level of expression of the avidin protein

The level of expression of avidin in each of the plant lines was quantitated usi chemiluminescence and expressed as percentage of total leaf protein. These levels a given in Table 1 below.

Table 1 The level of expression of avidin as % of total leaf protein, determined using the chemiluminescence method

Plant Line	Level of expression of avidin (% total leaf protein)
PLA2/2	0.07
PLA2/7	0.10
PLA2/9	0.07
PLA2/13	0.06
PLA2/20	0.065
PLA2/24	0.06

Mortality of PTM Larvae feeding on whole tobacco plants expressing the avidin gene

Trial i

Good recovery rates of larvae from both control and transgenic plants were obtained: 86% from controls and 76.7% from transformed plants. Fig. 10 clearly shows the high mortality PTM larvae after feeding for nine days on whole transgenic tobacco plants expressing the avidin gene compared to both non-transformed control plants and control plants transformed with, but not expressing, the pumpkin fruit chymotrypsin inhibitor (PFCI) gene.

The majority of dead larvae were recovered from mines where they had died at the "cutting face". A few (5% of dead larvae) were recovered from the surface of leaves, having generally left a mine close by. It is most likely that the majority of larvae not recovered had died in this way and had fallen off the leaves. Some mines were found without occupants. However, there was no evidence that larvae had started and abandoned mines.

PTM larvae undergo four instars during their development. In order to define the stage of development of the larvae at death, head capsule widths were measured using a micrometer eye-piece. All control larvae were alive and most were third instars. None of the larvae recovered on any of the plants expressing avidin had reached third instar before death and many had died during or just after the moult from first to second instar, as

evidenced by the fact that the ecdysed cuticle was still attached. This reflected results ir earlier trials with avidin incorporated into diet. Table 2 below gives a breakdown of instars on each plant line.

Table 2 Number of larvae at each instar recovered from transgenic tobacco plants expressing avidin in Trial i

Plant line	Neonates inoculated	Number of larvae at 1st instar	2nd instar	3rd instar	4th instar
NT control	20	0	1	18	0
JB3 control	30	0	0	23	1
PLA2/2	40	3	28	0	0
PLA2/7	40	4	23	0	0
PLA2/9	40	2	27	0	0
PLA2/13	40	1	25	0	0
PLA2/20	40	2	27	0	0
PLA2/24	40	4	25	0	0

Trial ii

Again there were good recovery rates of larvae from both control and transgenic plants: 88% from controls and 88.8% from transformed plants. Fig. 11 clearly reflects the results of the first trial showing high mortality of PTM larvae fed on whole transgenic tobacco plants expressing the avidin gene compared to those on control plants. In fact a total of only 4 live larvae were recovered from all avidin-expressing plants (<1.7% survival), whereas only 3 larvae had died on the control plants (94% survival).

Head capsule widths of larvae were measured and the number of recovered larvae at each instar is given in Table 3. As in the first trial, none of the larvae recovered from any of the plants expressing avidin had reached third instar before death and many had died during or just after the moult from first to second instar; again the ecdysed cuticle was still attached in several cases.

Table 3 Number of larvae at each instar recovered from transgenic tobacco p expressing avidin in Trial ii

Plant line	Neonates inoculated	Number of larvae at 1st instar	2nd instar	3rd instar	4th i
NT control	20	0	3	12	
JB3 control	30	0	1	24	
PLA2/2	40	34	5	0	
PLA2/7	40	25	9	0	
PLA2/9	40	30	3	0	
PLA2/13	40	26	11	0	
PLA2/20	40	25	5	0	
PLA2/24	40	30	3	0	

Conclusion

Total mortality of PTM larvae fed on tobacco plants expressing the avidin gene v have occurred if the trials had been continued beyond nine days; larvae that survive nine days were small, shrivelled and close to death as evidenced by their minimal resp when touched by a paintfine sable brush.

Avidin expressed in tobacco plants is highly toxic to PTM larvae and has definite pot in the development of pest resistant crop cultivars.

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All references are incorporated herein by reference.

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By the authorised agents
A. J Park & Son
Per

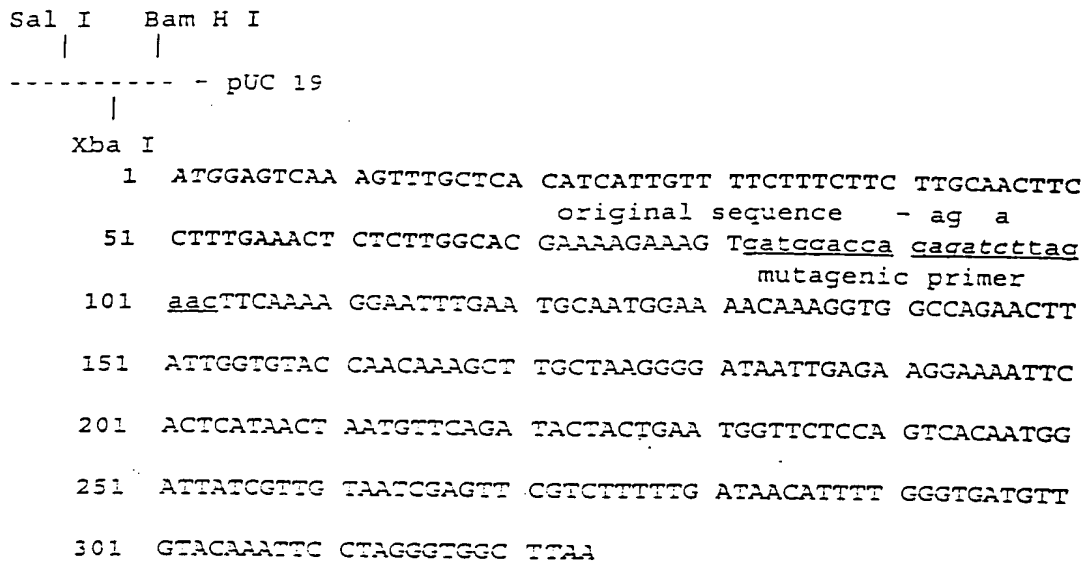

Figure 1

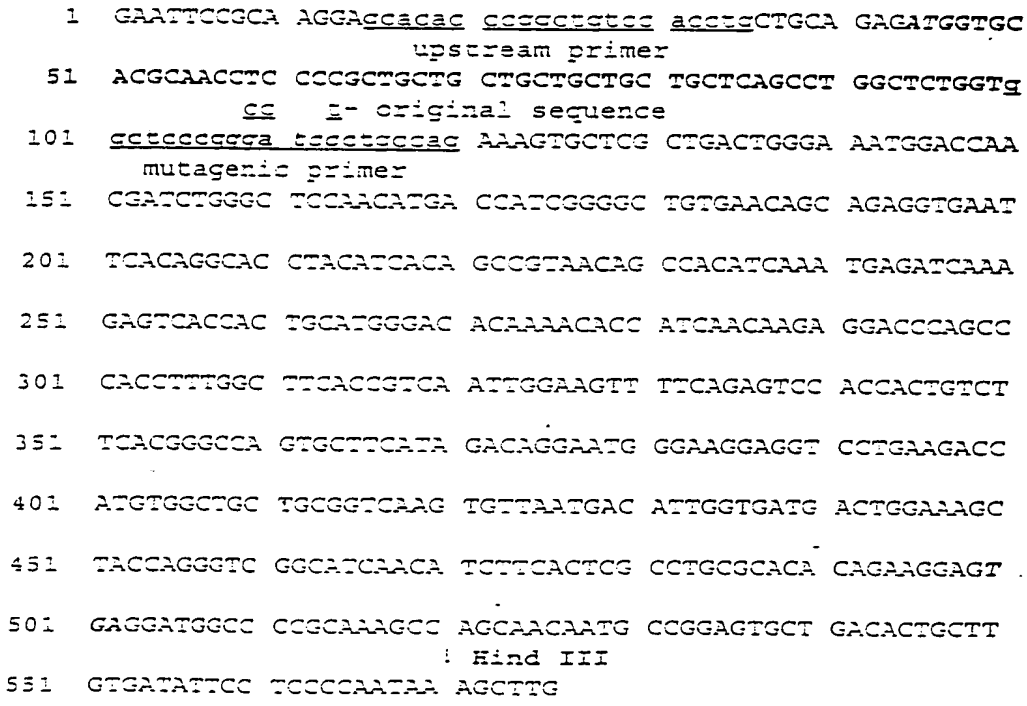

Figure 2

EcoR I

```
   1  GAATTCGCAT ATGGCTGAAG CTGGTATCAC CGGTACTTGG TACAACCAGC
  51  TGGGGTCTAC CTTCATCGTT ACCGCTGGTG CTGACGGTGC ACTGACCGGT
 101  ACTTACGAAA GCGCTGTTGG TAACGCTGAA AGCCGTTATG TTCTGACCGG
 151  TCGTTACGAC TCTGCTCCGG CTACCGACGG TTCTGGTACT GCTCTGGGTT
 201  GGACCGTTGC TTGGAAAAAC AACTACCGTA ACGCTCACTC TGCTACCACC
 251  TGGTCTGGCC AGTACGTTGG TGGTGCTGAA GCTCGTATCA ACACCCAGTG
 301  GCTGCTGACC TCTGGTACCA CCGAAGCTAA CGCTTGGAAA TCTACCCTGG
 351  TTGGTCACGA CACGTTCACC AAAGTTAAAC CGTCTGCTGC TTCTATCTAGA
```

Xba I

Figure 3

Sal I altered Bam H I*

---------- - pUC 19

Xba I

```
   1  ATGGATGTTC ACAAGGAAGT TAATTTCGTT GCTTACCTAC TAATTGTTCT
  51  TGGTAAGATT TTCCTTTACT CCTTTGTTTT AAAAAATAAA AAAACAAAAA
 101  AAATCTTGGT TTATACATAT ATATACACAC AAGTAGTTTT ATTTTTTTCC
 151  TTTATATTAT ATTTGTTGTA GGAATATTTC TACTTGTTAG CGTGGTGGAA
 201  CATGTTGATG CGAAGATCTG TACTAAAGAA TGTGGTAATC TTGGGTTTGG
 251  GATATGCCCA CGTTCAGAAG GAAGTCCGAA AAATCCCATA TGCATCAATT
 301  GTTGCTCAGG CTATAAGGGT TGTAATTATT ATAGTGTTTT CGGGAGATTT
 351  ATTTGCGAAG GAGAATCTGA CCTAAAAAAC CCAAAAGCTT GCCCCCTAAA
 401  TTGTGATACA AATATTGCCT ATTCAAGATG CCCCCATTCA GAAGGAAAAT
 451  CGCTAATTTA TCCCACCGGA TGTACCACAT GTTGCACAGG GTACAAGGGT
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Figure 4

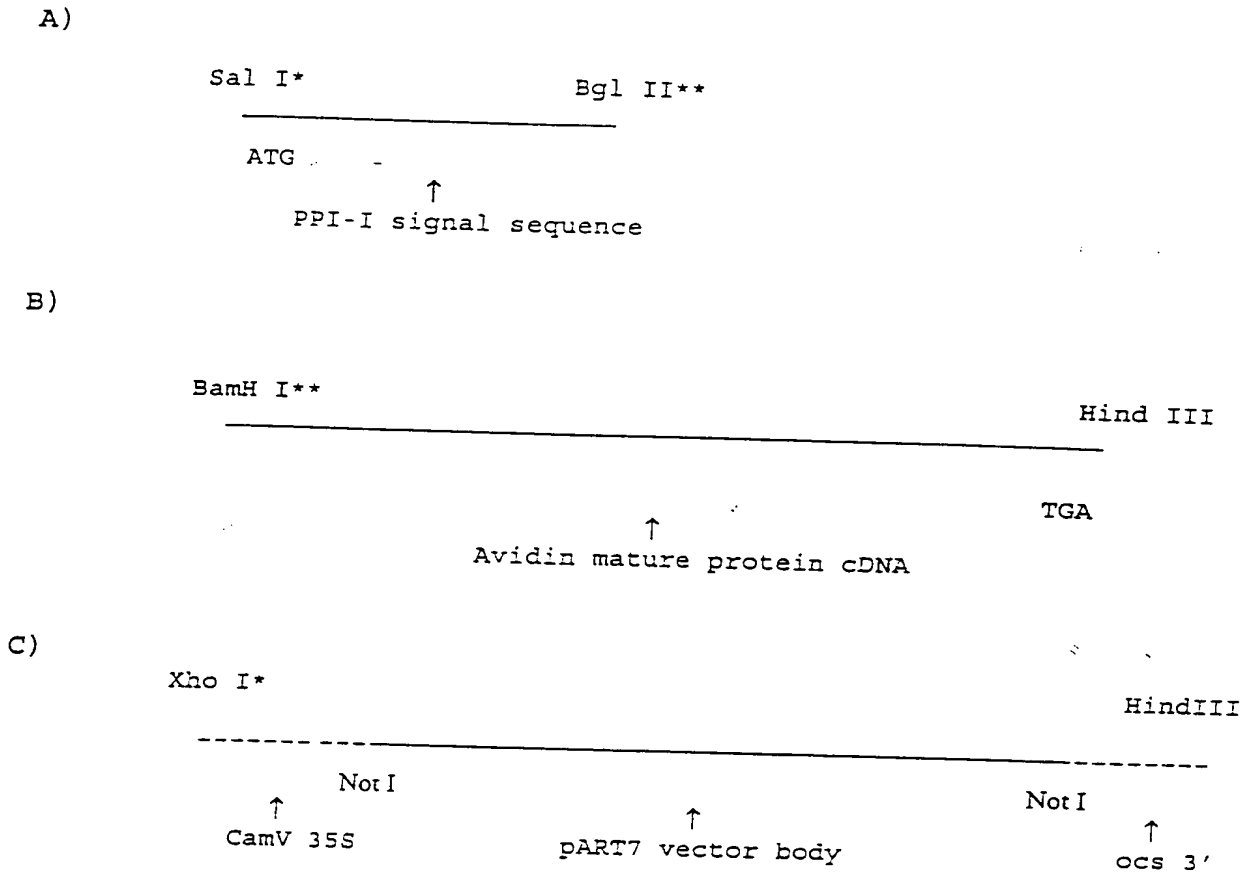
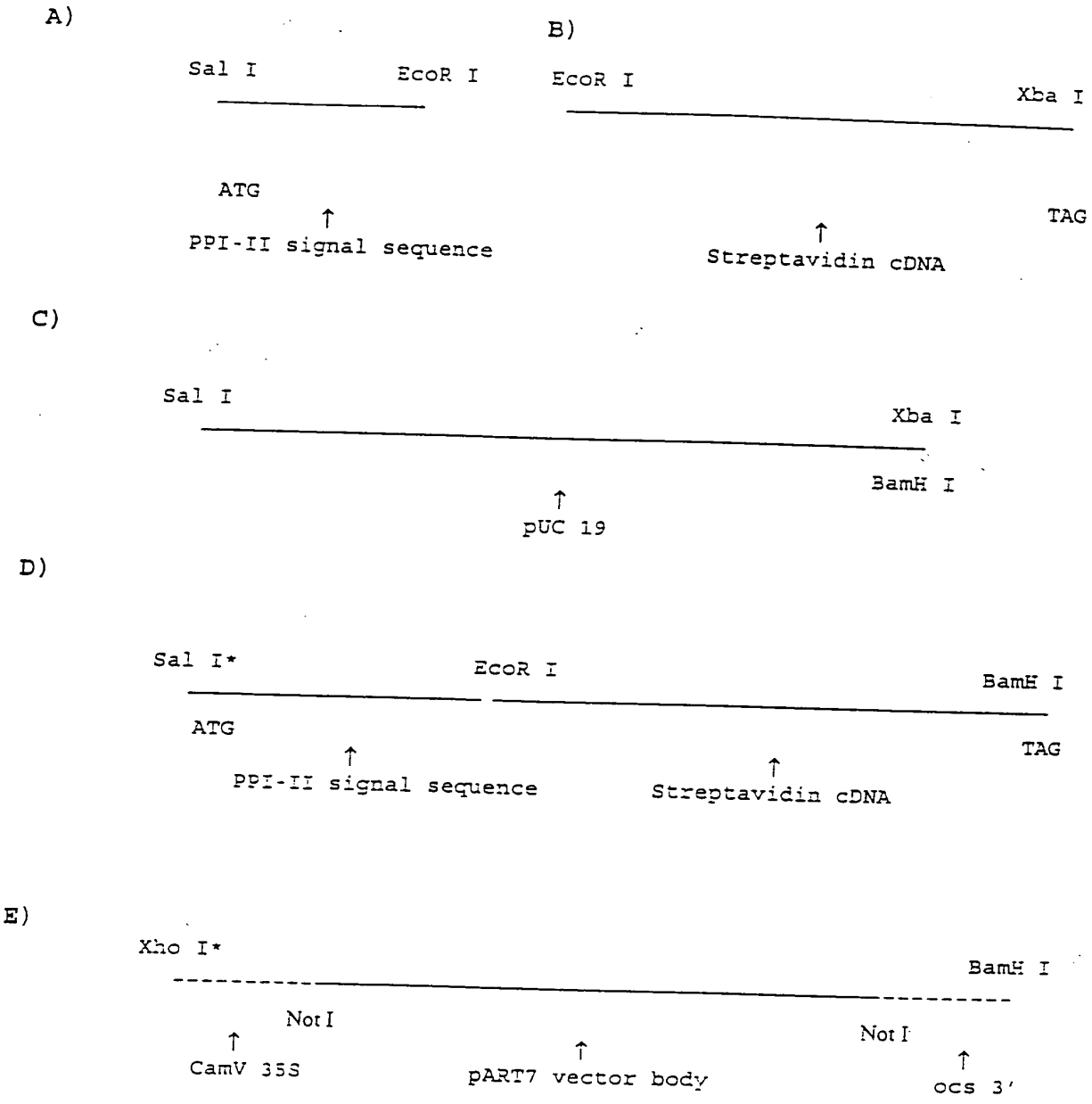

A)
Sal I*
Bgl II**
ATG
PPI-I signal sequence
B)
BamH I**
Hind III
TGA
Avidin mature protein cDNA
C)
Xho I*
HindIII
Not I
Not I
CamV 35S
pART7 vector body
ocs 3'

Figure 5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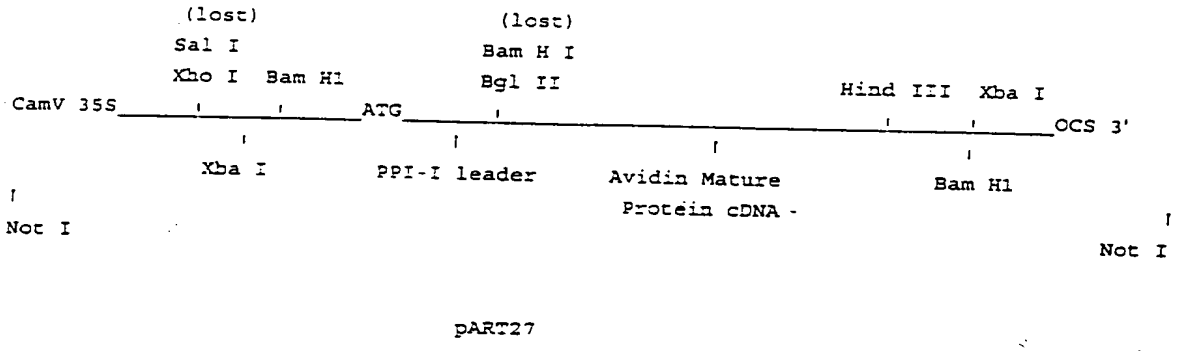
Sal I
EcoR I
ATG
PPI-II signal sequence
B)
EcoR I
Xba I
TAG
Streptavidin cDNA
C)
Sal I
Xba I
BamH I
pUC 19
D)
Sal I*
EcoR I
BamH I
ATG
TAG
PPI-II signal sequence
Streptavidin cDNA
E)
Xho I*
BamH I
Not I
Not I
CamV 35S
pART7 vector body
ocs 3'

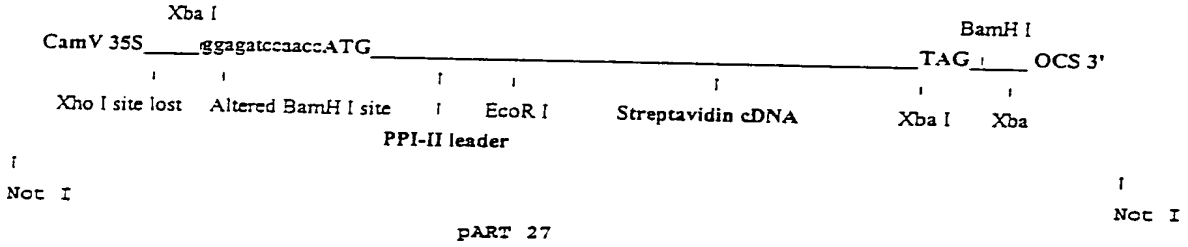
Figure 6

A)



pART27

B)



pART 27

Figure 7

A)

```
  1  ATGGAGTCAA AGTTTGCTCA CATCATTGTT TTCTTTCTTC TTGCAACTCC
 51  CTTTGAAACT CTCTTGGCAC GAAAAGAAAG TGATGGACCA GAGATCCCTG
101  CCAGAAAGTG CTCGCTGACT GGGAAATGGA CCAACGATCT GGGCTCCAAC
151  ATGACCATCG GGGCTGTGAA CAGCAGAGGT GAATTCACAG GCACCTACAT
201  CACAGCCGTA ACAGCCACAT CAAATGAGAT CAAAGAGTCA CCATTGCATG
251  GGACACAAAA CACCATCAAC AAGAGGACCC AGCCCACCTT TGGCTTCACC
301  GTCAATTGGA AGTTTTCAGA GTCCACCACT GTCTTCACGG GCCAGTGCTT
351  CATAGACAGG AATGGGAAGG AGGTCCTGAA GACCATGTGG CTGCTGCGGT
401  CAAGTGTTAA TGACATTGGT GATGACTGGA AAGCTACCAG GGTCGGCATC
451  AACATCTTCA CTCGCCTGCG CACACAGAAG GAGTGA
```

B)

cleavage site

```
  1  MESKFAHIIV FFLLATPFET LLARKESDGP EipARKCSLT GKWTNDLGSN
 51  MTIGAVNSRG EFTGTYITAV TATSNEIKES PLHGTQNTIN KRTQPTFGFT
101  VNWKFSESTT VFTGQCFIDR NGKEVLKTMW LLRSSVNDIG DDWKATRVGI
151  NIFTRLRTQK E*
```

Figure 8

A)

```
  1  ATGGATGTTC ACAAGGAAGT TAATTTCGTT GCTTACCTAC TAATTGTTCT
 51  TGGTAAGATT TTCCTTTACT CCTTTGTTTT AAAAAATAAA AAAACAAAAA
101  AAATCTTGGT TTATACATAT ATATACACAC AAGTAGTTTT ATTTTTTTCC
151  TTTATATTAT ATTTGTTGTA GGAATATTTC TACTTGTTAG CGTGGTGGAA
201  CATGTTGATG CGAAGATCTG TACTAAGAAT TCGCATATGG CTGAAGCTGG
251  TATCACCGGT ACTTGGTACA ACCAGCTGGG GTCTACCTTC ATCGTTACCG
301  CTGGTGCTGA CGGTGCACTG ACCGGTACTT ACGAAAGCGC TGTTGGTAAC
351  GCTGAAAGCC GTTATGTTCT GACCGGTCGT TACGACTCTG CTCCGGCTAC
401  CGACGGTTCT GGTACTGCTC TGGGTTGGAC CGTTGCTTGG AAAAACAACT
451  ACCGTAACGC TCACTCTGCT ACCACCTGGT CTGGCCAGTA CGTTGGTGGT
501  GCTGAAGCTC GTATCAACAC CCAGTGGCTG CTGACCTCTG GTACCACCGA
551  AGCTAACGCT TGGAAATCTA CCCTGGTTGG TCACNACACG TTCACCAAAG
601  TTAAACCGTC TGCTGCTTCT ATCTAG
```

B)

cleavage site

```
  1  MDVHKEVNFV AYLLIVLGIF LLVSVVEHVD AKICTKnshM AEAGITGTWY
 51  NQLGSTFIVT AGADGALTGT YESAVGNAES RYVLTGRYDS APATDGSGTA
101  LGWTVAWKNN YRNAHSATTW SGQYVGGAEA RINTQWLLTS GTTEANAWKS
151  TLVGHDTFTK VKPSAASI*
```

Figure 9

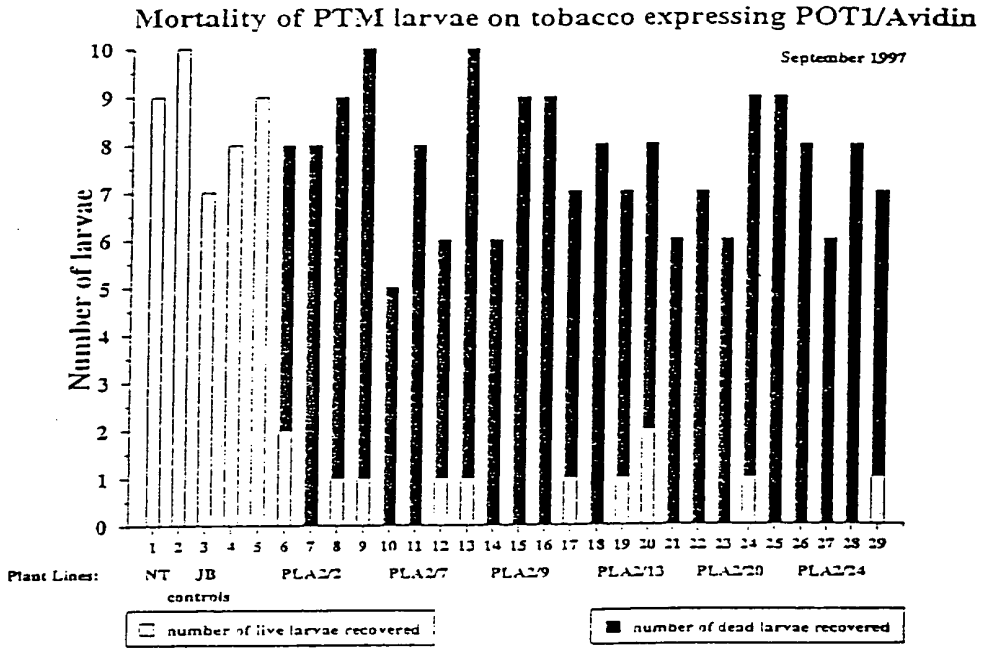


Figure 10

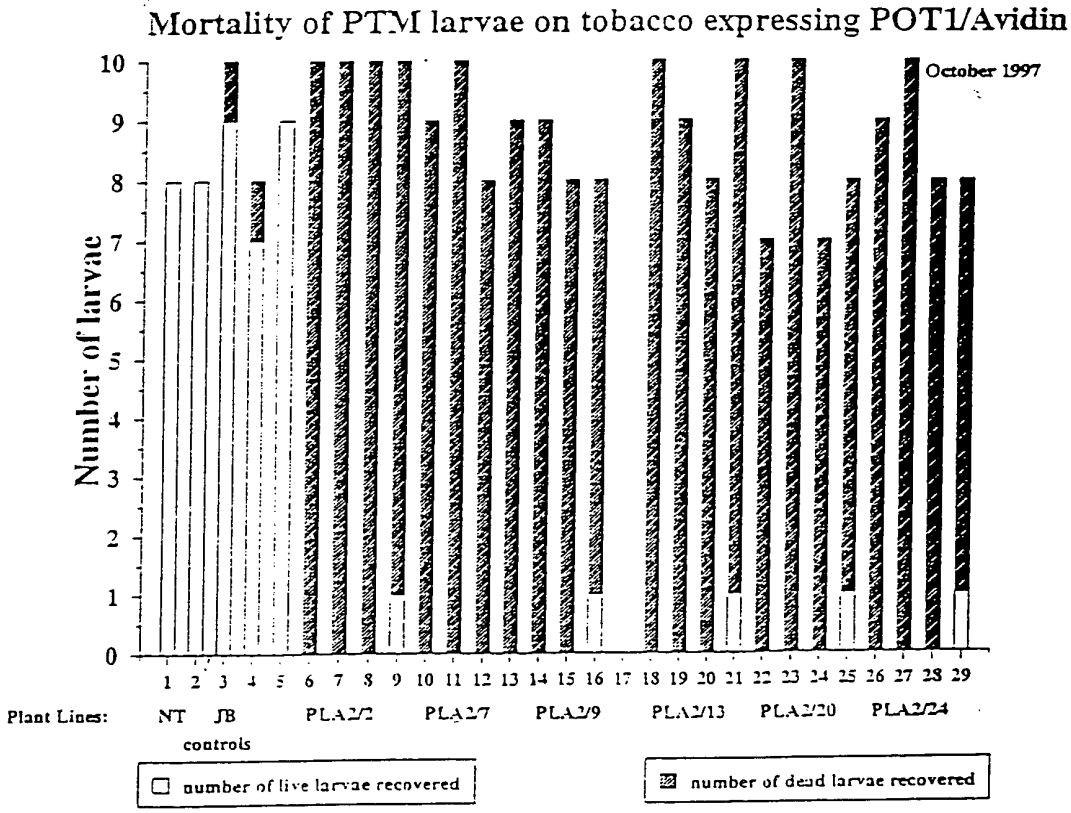


Figure 11

A)

```
   1  CCCTCCGTCC CCGCCGGGCA ACAACTAGGG AGTATTTTTC GTGTCTCACA
  51  TGCGCAAGAT CGTCGTTGCA GCCATCGCCG TTTCCCTGAC CACGGTCTCG
 101  ATTACGGCCA GCGCTTCGGC AGACCCCTCC AAGGACTCGA AGGCCCAGGT
 151  CTCGGCCGCC GAGGCCGGCA TCACCGGCAC CTGGTACAAC CAGCTCGGCT
 201  CGACCTTCAT CGTGACCGCG GGCGCCGACG GCGCCCTGAC CGGAACCTAC
 251  GAGTCGGCCG TCGGCAACGC CGAGAGCCGC TACGTCCTGA CCGGTCGTTA
 301  CGACAGCGCC CCGGCCACCG ACGGCAGCGG CACCGCCCTC GGTTGGACGG
 351  TGGCCTGGAA GAATAACTAC CGCAACGCCC ACTCCGCGAC CACGTGGAGC
 401  GGCCAGTACG TCGGCGGCGC CGAGGCGAGG ATCAACACCC AGTGGCTGCT
 451  GACCTCCGGC ACCACCGAGG CCAACGCCTG GAAGTCCACG CTGGTCGGCC
 501  ACGACACCTT CACCAAGGTG AAGCCGTCCG CCGCCTCCAT CGACGCGGCG
 551  AAGAAGGCCG GCGTCAACAA CGGCAACCCG CTCGACGCCG TTCAGCAGTA
 601  GTCGCGTCCC GGCACCGGCG GGTGCCGGGA CCTCGGCC
```

B)

```
   1  MRKIVVAAIA VSLTTVSITA SASADPSKDS KAQVSAAEAG ITGTWYNQLG
  51  STFIVTAGAD GALTGTYESA VGNAESRYVL TGRYDSAPAT DGSGTALGWT
 101  VAWKNNYRNA HSATTWSGQY VGGAEARINT QWLLTSGTTE ANAWKSTLVG
 151  HDTFTKVKPS AASIDAAKKA GVNNGNPLDA VQQ
```

Figure 12